Reference: *Biol. Bull.* **189**: 13–21. (August, 1995)

Oxidase Activity Associated with the Elevation of the Penaeoid Shrimp Hatching Envelope

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Abstract. When penaeoid shrimp spawn into seawater, the ova elevate a hatching envelope (HE) within 30–50 min. By 60 min after spawning, the bilayered HE is completely formed. In other animal systems, peroxidatic enzymes are responsible for the hardening of the extra-embryonic coat. In this study, observations are made consistent with the involvement of an oxidase in the assembly of the shrimp HE. As observed by electron microscopy, eggs of *Sicyonia ingentis* and *Trachypenaeus similis* spawned in seawaters containing peroxidase inhibitors had abnormally assembled HEs compared to control eggs in seawater. Dihydrotetramethylrosamine, an oxidase-sensitive fluorescent dye, supravitally stained the cortex of *S. ingentis* eggs at the time of initial HE formation. The HE fluoresced from elevation (40–50 min postspawn in *S. ingentis*) until 60–70 min postspawn. By first cleavage (90–120 min postspawn), HE staining was no longer visible, although staining persisted in the egg cortex. In eggs treated with the peroxidase inhibitors 3-amino-1,2,4-triazole or sodium sulfite, the egg cortex fluoresced, but no fluorescence appeared in the HE before, during, or after its elevation.

Introduction

Formation of extracellular egg coats has been the subject of studies especially in frogs (for review, Schmell *et al.*, 1983), sea urchins (for review, Schuel, 1978, 1985; Kay and Shapiro, 1985), and crustaceans. The crab and lobster egg coats were described by Goudeau and her colleagues (Goudeau and Becker, 1982; Goudeau and Lachaise, 1980, 1983; Talbot and Goudeau, 1988), and Pillai and Clark (1987, 1988, 1990) described the elevation and formation of the hatching envelope of the penaeid shrimp *Sicyonia ingentis*.

The presence of an ovoperoxidase enzyme has been demonstrated as crucial in the assembly of the sea urchin fertilization envelope (FE) (Kay and Shapiro, 1985, for review). Peroxidases catalyze reactions in which hydrogen peroxide serves as a substrate that, when bound to peroxidase, oxidizes other substances much more rapidly than alone (Kiernan, 1990). Ovoperoxidase in sea urchins forms a complex with the protein proteoliaisin and is incorporated into the FE as it catalyzes di- and tri-tyrosine linkages. This hardens the sea urchin FE mechanically so that it is resistant to sperm proteases and environmental stress. Extra-embryonic coat assembly involving di- and tri-tyrosine linkages mediated by an ovoperoxidase-proteoliaisin complex has been described in detail (for review, Kay and Shapiro, 1985, 1987; Weidman *et al.*, 1985, 1987; Somers *et al.*, 1989). In fish, Kudo *et al.* (1988) demonstrated a peroxidatic reaction in the fertilized fish egg chorion. This is believed to be part of the hardening reaction in the chorion necessary to provide a microenvironment for the developing embryo (Kudo and Inoue, 1986, 1989; Kudo and Teshima, 1991; Kudo, 1992).

These peroxidatic reactions were visualized by substrate localization using 3,3-diaminobenzidine (DAB) (Daems *et al.*, 1964; Katsura and Tominaga, 1974; Klebanoff *et al.*, 1979; Kudo *et al.*, 1988; Green *et al.*, 1990). Ovoperoxidase inhibitors such as 3-amino-1,2,4-triazole (ATA) or sodium sulfite have been used to prevent normal assembly of sea urchin FE (Veron *et al.*, 1977; Showman and Foerder, 1979). In the presence of inhibitors, lack of incorporation of the ovoperoxidase enzyme is implicated

Received 16 September 1994; accepted 24 May 1995.

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Abbreviations: 3-amino-1,2,4-triazole (ATA); 3,3-diaminobenzidine (DAB); artificial seawater (ASW); hatching envelope (HE); scanning electron microscopy (SEM); transmission electron microscopy (TEM); fertilization envelope (FE); dihydrotetramethylrosamine (DHTMR); perivitelline space (PVS).

by an absence of DAB precipitation staining (Katsura and Tominaga, 1974; Klebanoff *et al.*, 1979; Green *et al.*, 1990). Although ovoperoxidase has been demonstrated in the cortical granules in mammals, other enzymes are believed to account for the change in zona pellucida permeability during the zona reaction (Wasserman, 1987; Bleil and Wasserman, 1980).

In eggs of the penaeoid shrimp *Sicyonia ingentis* and *Trachypenaeus similis*, the hatching envelope (HE) is elevated 30–50 min after spawning in seawater. This elevation is the result of exocytosis of at least two distinct types of cortical vesicles in *S. ingentis* (Pillai and Clark 1988; 1990) and *T. similis* (Lynn *et al.*, 1991; Glas, 1994). Assembly results in an HE with a thin, electron-dense outer layer and a thick, more electron-translucent inner core. When peroxidase inhibitors were added to the spawning media, abnormal hatching envelopes elevated (Lynn *et al.*, 1993) indicating that a peroxidatic reaction may be necessary for normal HE assembly. However, attempts to localize a peroxidase with DAB did not show significant staining. This apparently conflicting evidence suggests the presence of an oxidatic enzyme, but not necessarily ovoperoxidase.

Materials and Methods

Gamete collection

Animals were collected as previously described by Pillai *et al.* (1988) and Lynn *et al.* (1991, 1992, 1993). Eggs were collected in 70 × 50 mm crystallizing dishes containing artificial seawater (ASW) (Cavanaugh, 1956). The dishes were gently swirled for 10 min postspawning to prevent clumping of the eggs.

Inhibitor and localization substrates

The peroxidase inhibitors ATA or sodium sulfite were added to dishes of eggs in ASW 10 min after spawning to give a final concentration of 100 μM ATA (Lynn *et al.*, 1993) or 20 mM sodium sulfite in seawater. Hatching envelope elevation was monitored with light microscopy.

Samples for light microscopy were removed from the spawning dishes at intervals to correct for temperature changes from the microscope light. Transmission electron microscopy (TEM) samples were removed and processed by fixation, osmication, dehydration (Lynn *et al.*, 1992), infiltration, and embedding in modified Spurr's resin (Spurr, 1969). Sections were double stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Scanning electron microscopy (SEM) samples were processed through dehydration, then critical-point dried, sputter-coated, and viewed.

For fluorescent localization of enzyme activity, a peroxidase-sensitive, rhodamine analog dye, dihydrotetramethylrosamine (DHTMR) (Molecular Probes, D-638), was added directly to spawning dishes containing *S. ingentis* eggs. The dye was in an aqueous stock solution of 2 mg/ml of which 100 μl per 100 ml egg suspension was used. An FITC filter cube with excitation at 485 nm and transmission at 510 nm was used to observe eggs for fluorescence.

Localization of peroxidase activity with DAB as a substrate was performed as described by Klebanoff *et al.* (1979). Samples were removed from the spawning dish and washed in a solution of 0.1 M TRIS–0.45 M NaCl, pH 8.0, to remove salts that might interfere with the reaction. The wash solution was replaced with a reaction solution (2 mg/ml of 3,3-diaminobenzidine, 0.01% H_2O_2 in 0.1 M Tris–0.45 M NaCl, pH 8.0), and the reaction was allowed to proceed for 10 min. The reaction was stopped by the addition of two times the volume of 5.0% glutaraldehyde/1.6% formaldehyde fixative in 0.1 M Tris-buffer, pH 8.0. Samples were then processed for TEM. Both stained and unstained sections were observed.

Permeability of the hatching envelope

To ascertain permeability of the HE, fluorescently tagged dextrans (500 μg/ml egg suspension) (Sigma) were added to the ASW and ATA seawater media after the hatching envelope was visible. Eggs were observed with epifluoresence until 90 min postspawn. A flow-through chamber was used on an inverted microscope to allow changing the solution without agitating the eggs. Dextrans of 4400, 10,000, 40,000, 76,000, and 155,000 molecular weight, conjugated to fluorescein isothiocyanate (FITC-dextran) or tetramethylrhodamine isothiocyanate (TRITC-dextran) probes were used. Eggs were incubated in the presence of the dextran at room temperature. Eggs were transferred to a flow-through chamber, which was purged two times with seawater. The eggs were observed for the absence of fluorescence within the perivitelline space (PVS) by using FITC or TRITC excitation and barrier filters on a Nikon diaphot inverted microscope. Dye exclusion was indicated by decreased fluorescence compared to the medium outside the hatching envelope in the PVS when eggs were observed at an equatorial focus.

Enzyme assays

Enzyme assays were performed on supernatants from the spawned eggs to detect ovoperoxidase secretion from the egg. The guaiacol assay was used as described by Deits *et al.* (1984) with 28 mM guaiacol, 1.0 mM H_2O_2, and 50–500 μl supernatant from settled eggs in the spawning dishes. Supernatant from a dish with spawned eggs was assayed at 10, 30, 45, and 60 min postspawn. Protein assays were as described by Lowry *et al.* (1951).

Results

Historical descriptions

For clarity, a brief description of hatching envelope (HE) formation in *S. ingentis* and *T. similis* is reiterated here. Eggs spawned from penaeoid shrimp release a jelly coat upon contact with seawater (Pillai and Clark, 1987; Lynn *et al.*, 1991). The first cortical vesicle exocytosis occurs about 30–35 min postspawn in *S. ingentis*, and the products interact with a surface coat to form the outer, electron-dense layer of the HE (Pillai and Clark, 1988). The HE is elevated by 40–50 min postspawn. HE elevation is reported to be independent of fertilization (Clark *et al.*, 1980; Pillai and Clark, 1987). In *T. similis*, the first exocytosis also occurs about 30 min postspawn. However, the second exocytosis follows more rapidly so that the HE is elevated by 40 min postspawn (Glas, 1994). Formation of the first polar body occurs 5 to 10 min before the HE is apparent. The second polar body appears at 30–45 min postspawn beneath the elevating HE, and both polar bodies remain throughout HE elevation. During this period, granular material is observed accumulating in the PVS. At 90 min postspawn, the HE is refractile and an expansive PVS is visible. Transmission electron micrographs reveal an HE with a distinct bilayered appearance.

The criteria for successful assembly and elevation of the HE were based on the morphology of the envelope. These included the continued elevation of the HE without collapse and a bilayered appearance. The extra-embryonic envelopes remain around the zygote until the time of hatching, about 24 h later.

Assessment of peroxidase activity

In *S. ingentis* eggs spawned into ASW, a distinct PVS was visible at 75 min postspawn (Fig. 1), separating the oolemma from the elevated HE. With TEM, the HE appeared as a well-formed structure with flocculent material juxtaposed on the interior of an electron-dense outer layer. Remnants of the jelly layer remained outside the HE (Fig. 2). Materials within the PVS were seen in close association with the oolemma or the thickening HE. With higher magnification, the bilayered configuration of the HE was evident as a distinct electron-dense outer layer and a flocculent, more electron-translucent inner layer (Fig. 3). The exterior of the egg envelope had no noticeable ridges or marks.

S. ingentis eggs treated with ATA elevated HEs by 45 min postspawn. Envelope elevation in these samples often preceded that in the control samples by 5 min, and initial elevation was frequently greater than in control egg HEs. At 75 min postspawn, inhibitor-treated eggs had envelopes that were more refractile and less birefringent than the control eggs, and the envelopes often collapsed to the oolemma (Fig. 4). The envelopes were very fragile, were often wrinkled and folded, and were easily removed (Lynn *et al.*, 1993; Glas, 1994). Transmission electron micrographs of envelopes and eggs of ATA-treated samples revealed no significant differences between control and treated eggs in the appearance of the PVS or the release of cortical vesicles (compare Figs. 2 and 5). HEs of eggs in ATA, however, showed a dense outer layer, but the more electron-translucent layer was absent or poorly developed (Fig. 6). This resulted in a thinner envelope than seen in the controls (Figs. 3 and 6).

In *S. ingentis*, even at 75 min postspawn, definitive DAB localization was absent in the hatching envelope in ASW (data not shown). The intense black precipitant indicative of peroxidase activity in other systems such as the sea urchin FE was not present (see Klebanoff *et al.*, 1979). Similarly, DAB staining was also not apparent in the elevated HE of *T. similis* eggs.

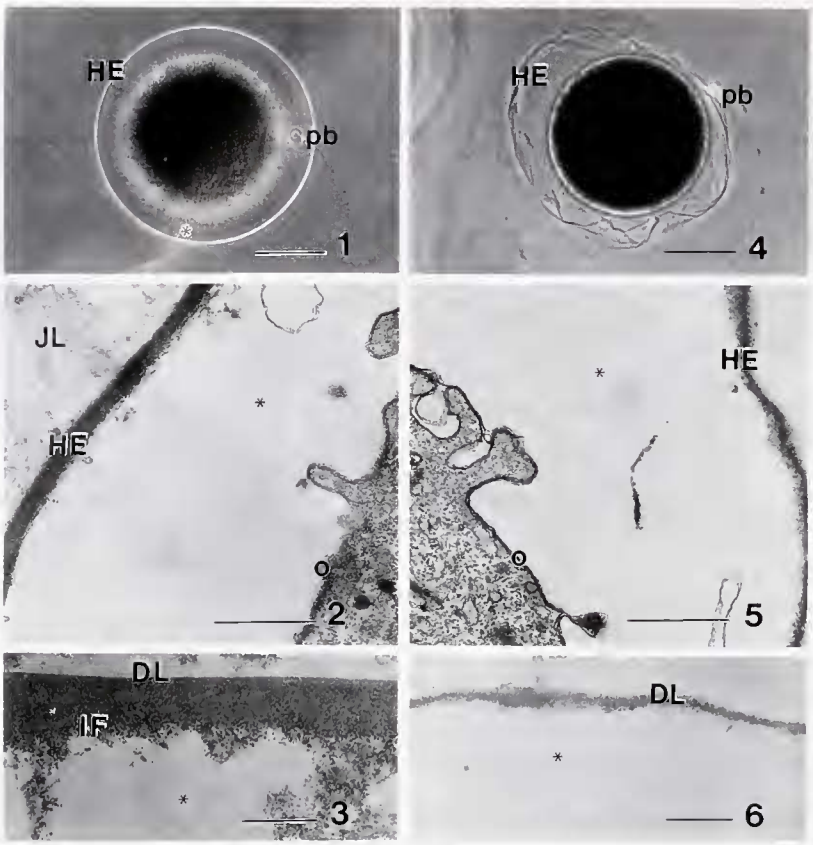
In *T. similis* eggs, the HE appeared as a distinct refractile coat around the egg by 40 min postspawn and remained so at 90 min postspawn (Fig. 7). The intact HE prevented observation of the oolemma in scanning electron micrographs (Fig. 8). Handling and dehydration techniques caused the envelope to collapse, so that large folds in the HE were observed with SEM. At higher magnifications, small ridge-like contours on the exterior of the envelope were visible. These ridges were arranged in distinctive polygonal patterns encompassing areas of smooth envelope (Fig. 9).

In transmission electron micrographs of *T. similis* eggs at 90 min postspawn, the HE appeared as a bilayered structure (Fig. 10). The ridge-like contours seen with SEM correlated with cone-shaped protrusions of electron-dense material (Fig. 10). This material appeared continuous with the electron-dense outer layer of the hatching envelope. A flocculent, less electron-dense layer formed interiorly to the outer layer, and was three to four times the thickness of the outer dense layer (Fig. 10).

T. similis eggs treated with 100 μm ATA at 10 min postspawn elevated HEs about 30–35 min postspawn. By 90 min postspawn, eggs incubated in ATA had envelopes that appeared less refractile (Fig. 11). Frequently, the granular material observed in the PVS was coarser and more abundant than in control eggs. When 90-min-postspawn eggs were observed with SEM, a ridge-like pattern was distinguishable (Fig. 12), but was much less prominent (Fig. 13) than in the control eggs (Fig. 9).

At 90 min postspawn, eggs treated with ATA appeared to have a thinner HE that consisted of the electron-dense layer (Fig. 14) with the flocculent, more electron-translucent layer absent or greatly reduced when examined with TEM. The inhibitor-treated HEs often folded on themselves, so that, in section, the ridges sometimes appeared to be on the "inside" of the envelope (Fig. 14).

When *S. ingentis* eggs were treated with the oxidase-sensitive fluorescent dye dihydrotetramethylrosamine



Figures 1–6. *Sicyonia ingentis* eggs. HE, hatching envelope; PVS, perivitelline space; ATA, 3-amino-1,2,4-triazole.

Figure 1. With phase microscopy, eggs in artificial seawater show a distinctive refractile HE surrounding the PVS (*). The second polar body (pb) is visible within the PVS. Bar equals 100 μm.

Figure 2. With transmission electron microscopy, eggs in artificial seawater show the jelly layer (JL) outside of the HE (HE). The PVS (*), separating the HE and oolemma (O), contains materials that may be added to the HE. Bar equals 1 μm.

Figure 3. At higher magnification, the bilayered structure of the HE is apparent. The outer dense layer (DL) forms a smooth exterior while the inner flocculent layer (IF) appears to be incorporating more material from the PVS (*). Bar equals 0.25 μm.

Figure 4. Eggs in 3-amino-1,2,4-triazole (ATA) seawater have HEs (HE) that often collapse. The first polar body (pb) is visible outside of the collapsed HE. Bar equals 100 μm.

Figure 5. With transmission electron microscopy, eggs in ATA seawater show HEs (HE) that do not have the structural bilayered appearance of those in control eggs. The inner electron translucent layer is missing. The PVS (*) separates the oolemma (O) from the HE. Bar equals 1 μm.

Figure 6. Higher magnification shows that the ATA-treated HEs are thinner and do not appear to have the flocculent inner layer attached to the dense layer (DL). Bar equals 0.25 μm.

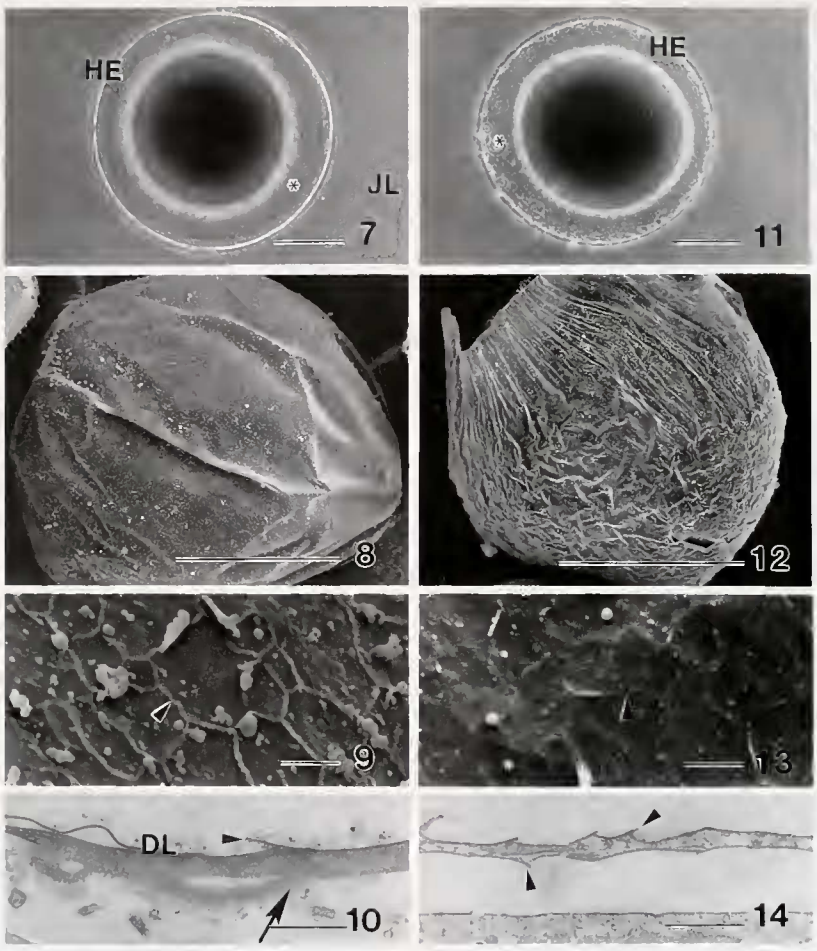
(DHTMR) (Whitaker *et al.*, 1991), no fluorescence was visible in the egg before HE elevation. Sperm attached to the egg surface were visibly fluorescent (Fig. 15a, b). A fluorescent band appeared in the cortex of the egg as the HE became visible (Fig. 16a, b). A thin, fluorescent outline of the HE appeared after HE elevation. The HE fluorescence intensified briefly and remained in the region of the newly elevated HE until about 60 min postspawn (Fig. 17a, b). By the two-cell stage, the fluorescence in the HE was no longer detectable (Fig. 18a, b).

The cortex of eggs incubated in ATA was fluorescent immediately before HE elevation, as in controls. However, unlike the controls (Figs. 15a, b; 16a, b; 17a, b), eggs in ATA showed no fluorescence associated with the HE during and after elevation (Fig. 19a, b).

Eggs incubated in another peroxidase inhibitor, sodium sulfite, showed no cortical fluorescence immediately before HE elevation. Continued monitoring showed no fluorescence by 60 min postspawn in the elevated HE (Fig. 20a, b).

Hatching envelope permeability

S. ingentis eggs were incubated with fluorescently labeled dextrans of a range of sizes to examine the perme-



Figures 7–14. *Trachypenaeus similis* eggs. HE, hatching envelope; PVS, perivitelline space; ATA, 3-amino-1,2,4-triazole.

Figure 7. Eggs in artificial seawater show a jelly layer (JL) outside of the refractile HE (HE) that surrounds the PVS (*). Bar equals 100 μm.

Figure 8. Scanning electron microscopy shows the HE covering the developing zygote to act as an environmental barrier. The HE collapses during processing, resulting in some wrinkling of the envelope. Bar equals 100 μm.

Figure 9. Higher magnification of the HE shows an exterior series of ridges (arrowhead) not seen in *Sicyonia ingentis* egg HEs. The ridges form octagonal patterns on the surface of the HE. Bar equals 1 μm.

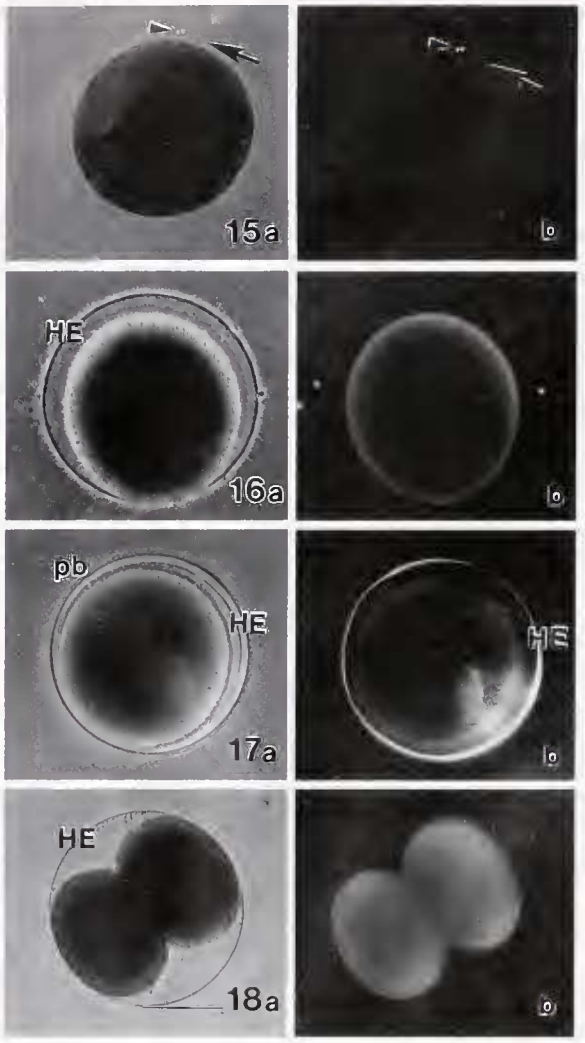
Figure 10. Transmission electron microscopy shows the distinct bilayered HE with the ridges appearing to be constructed of the electron-dense material (DL). The arrowhead indicates a ridge now seen in cross section. Note the ring material in the PVS associated with the flocculent inner layer (large arrow). The flocculent layer is three to four times the thickness of the outer dense layer. Bar equals 0.5 μm.

Figure 11. Phase microscopy of an egg in ATA shows a less refractile HE (HE) surrounding the PVS (*). Bar equals 100 μm.

Figure 12. Scanning electron microscopy of an egg in ATA seawater shows an egg with more folds than in ASW. The collapse of the HE causes the envelope to be folded on itself. Bar equals 100 μm.

Figure 13. At higher magnification, the ridges (arrowhead) are not as prominent as in the control eggs. The height of the ridges appears lower than in normal eggs. Bar equals 1 μm.

Figure 14. Transmission electron microscopy shows that the envelope is folded so that ridges (arrowheads) appear to be on the interior and exterior of the HE. The electron-dense layer is present, but the electron-translucent layer is greatly reduced. Bar equals 0.5 μm.



Figures 15–18. A time series of *Sicyonia ingentis* eggs in artificial seawater shows the elevation of the hatching envelope (HE) under light microscopy (a) and labeling of the HE with an oxidase sensitive fluorescent dye, dihydrotetramethylrosamine (b).

Figure 15. (a) Normal egg before elevation at 35 min postspawn. Two sperm are visible on the exterior of the egg (arrowhead). Arrow indicates first polar body. (b) No fluorescence is visible in the cortex; however, the sperm are fluorescent (arrowhead). The first polar body (arrow) can also be seen.

Figure 16. At 47 min postspawn, the HE has begun to lift from the oolemma. (a) The HE (HE) is separating from the oolemma and has extranumerary sperm on its exterior. (b) The cortex can be seen to react with DHTMR.

Figure 17. By 60 min postspawn, the HE is fully formed. (a) A normal HE (HE) with the second polar body (pb) visible in the perivitelline space. (b) The HE fluoresces brightly, as does the cortex of the egg. The polar body does not fluoresce.

Figure 18. At 100 min postspawn, the two-cell stage can be seen. (a) The cells are visible within the elevated HE (HE). (b) The cortex still fluoresces, but the HE does not. Bar equals 100 μm.

ability of the HE (Table 1). Based on the relative fluorescent intensity inside *vs.* outside the PVS at an equatorial focus, both the control and ATA-treated eggs had fluorescent dextrans within the PVS when incubated with the 4400 KDa sugar. When incubated with the 10,000 KDa dextran, the ATA-treated eggs contained fluorescent dextrans within the PVS, but eggs in ASW did not. The entry of the dextrans was variable in the ATA-treated eggs with 40,000 and 76,000 Da dextrans, although the control eggs revealed no fluorescent dextrans within the PVS (Table 1). Even though extreme care was used, the presence of high molecular weight fluorescent dextrans in the PVS of ATA-treated eggs may have been due to damage of the envelope allowing the dextrans to enter the PVS.

Enzyme assays

A spectrophotometric assay using the colorometric substrate guaiacol (Foerder and Shapiro, 1977) failed to indicate peroxidase activity in the supernatant within 3 min at 10, 30, 45, or 60 min postspawn. These time points encompass the period before, during, and after HE elevation.

Discussion

Histochemical localization and identification of peroxidase using DAB staining is well documented (Kay and

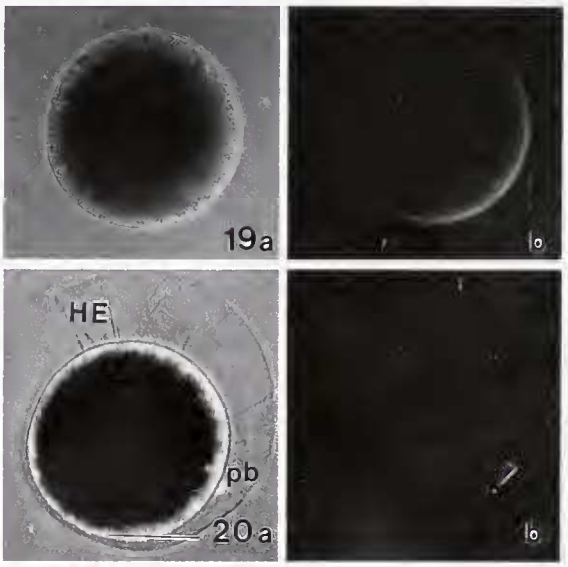


Figure 19. *Sicyonia ingentis* eggs were treated with the peroxidase inhibitor 3-amino-1,2,4-triazole and then incubated with DHTMR. (a) In the inhibitor, the eggs elevate the hatching envelope (HE), which then frequently collapses back to the egg surface. (b) With DHTMR, the cortex can be seen to fluoresce weakly on one side of the egg and is not consistent with fluorescence in the HE (compare with same time point of controls in Fig. 17a, b).

Figure 20. *Sicyonia ingentis* eggs were also treated with sodium sulfite, a peroxidase inhibitor, and then incubated with DHTMR. (a) The hatching envelope (HE) is elevated, but collapses around the egg as seen with light microscopy. The second polar body (pb) is visible. (b) Fluorescent microscopy shows no labeling of the HE or cortex although the sperm shows fluorescence (arrowhead). Bar equals 100 μm.

Table I

Presence of fluorescent dextrans in the perivitelline space after hatching envelope elevation

Molecular weight	Control (artificial seawater)	Treated (3-amino-1,2,4-triazole + seawater)
4,400	−, ½+ ½−	+,+
10,000	−	+
40,000	−	−
76,000	−,−,−	−,+,−
155,000	−,−	+,−

Each score represents a separate trial of 10 or more visibly undamaged eggs.

+ not excluded from the perivitelline space; − excluded from the perivitelline space.

½+ 50% had fluorescence in the perivitelline space; ½− 50% excluded fluorescence.

Shapiro, 1985, for review; Kiernan, 1990; Green *et al.*, 1990). These studies report intense black staining as indicative of the peroxidase reaction in sea urchin eggs and mammalian tissue. DAB staining due to peroxidatic activity has been reported in the FEs of the sea urchins *Hemicentrotus pulcherrimus, Temnopleurus toreumaticus, Strongylocentrotus purpuratus,* and *Lytechinus pictus* (Katsura and Tominaga, 1974; Klebanoff *et al.*, 1979; Green *et al.*, 1990). Using DAB, Kudo *et al.* (1988) also demonstrated peroxidase activity in the vitelline envelope of unfertilized fish eggs and in the chorion and micropylar region of fertilized fish eggs. In addition, DAB reactivity remains detectable over an extended time (Klebanoff *et al.*, 1979; Kay and Shapiro, 1985, for review; Green *et al.*, 1990). Heavy precipitation indicative of peroxidase activity was not visualized in the HE of eggs from either *S. ingentis* or *T. similis* by using the DAB methodology. Staining was not observed at any of the time points sampled (30, 45, 75, or 90 minutes postspawn).

The peroxidase inhibitors ATA and sodium sulfite are believed to interfere with tyrosine cross-linking by oxidative inhibition of the enzyme or enzymes responsible for FE elevation and transformation (Kay and Shapiro, 1985, for review; Kay and Shapiro, 1987; Lynn *et al.*, 1988; Green *et al.*, 1990). Inhibitor-treated sea urchin eggs have FEs that are thinner and less refractile than the normal envelopes. Treatment of penaeid shrimp eggs with the peroxidase inhibitors ATA and sodium sulfite results in "soft" HEs that are less refractile and tend to collapse. The morphology of the envelope shows a thinner envelope that lacks the bilayered appearance. These inhibitors are not detrimental to the development of the shrimp egg (Lynn *et al.*, 1993).

In contrast, interference by "peroxidase" inhibitors with normal HE assembly is also implicated by changes in HE permeability as exhibited by fluorescently labeled dextrans. In these experiments, the HE is more permeable to substances of 10,000 and 40,000 kDa in peroxidase-inhibited medium than in artificial seawater. The variability of exclusion in the higher ranges may be due to other influences including time of dextran addition, damage to the envelopes, or possibly, whether fertilization had taken place. Variation due to damage of the envelopes was minimized by developing a system with minimum handling of the eggs. Further implications of differences in HE permeability due to sperm interaction were not considered during this study since envelope elevation and formation were previously reported to be independent of fertilization (Pillai and Clark, 1987).

The paradox of these findings resulted in the use of a fluorescent probe, dihydrotetramethylrosamine (DHTMR), a rhodamine analog. DHTMR reacts with oxidizable substrates and is pH independent in its absorption and emission (Whitaker *et al.*, 1991). Whitaker *et al.* (1991) describe this compound as a neutral, lipophilic substrate for horseradish peroxidase and hydrogen peroxide. An advantage of the dye is that it can be used with *in vitro* preparations, thereby eliminating the use of fixatives and the accompanying interference by aldehyde groups. The cytoplasm of spawned eggs placed in this dye fluoresces, indicating the presence of an oxidative capacity. Similar fluorescence is reported in mammalian and protozoan systems in relation to phagocytosis and the immune response (Whitaker *et al.*, 1991). The dye reaction in shrimp eggs may be indicative of several reactions. These reactions could include a peroxidase, a hydroxide, or superoxide-based change that may correspond to the "respiratory burst" in sea urchin eggs (Turner *et al.*, 1985; Heinecke and Shapiro, 1989; Epel, 1990, for review) or respiratory activity associated with mitochondria. Thus, it appears that DHTMR may react with one or more substrates in the shrimp egg, but the substrate cannot be defined as ovoperoxidase. In shrimp, bound sperm also fluoresced, a result in keeping with observations by Lindsay and Clark (1992) of a pH (hydroxide) change involved in sperm acrosomal filament formation. It is unclear why all sperm that were attached to eggs did not fluoresce, but there did not appear to be any correlation to the fertilizing sperm. Since this was not the main point of this study, further investigations will be required to resolve this interesting phenomenon.

Comparison of the amino acid composition of the sea urchin FE (Foerder and Shapiro, 1977) with the amino acid composition of the shrimp HE (Pillai and Clark, 1990) reveals that the relative abundance of tyrosine residues is considerably lower in the shrimp HE than in the sea urchin FE. Because tyrosine is a major substrate for ovoperoxidase activity, lack of an abundant supply may suggest there is not a major role for an ovoperoxidase in shrimp eggs. Assays for the di- and tri-tyrosine components in the shrimp HE have not been performed.

Sufficient ovoperoxidase is released by sea urchin eggs to allow quantitative analysis of the supernatant (Deits *et al.*, 1984; Green *et al.*, 1990). In peneaoid shrimp, however, if an ovoperoxidase is released following spawning, the quantities are insufficient to detect between spawning and 90 minutes postspawn. Although HE assembly could be acting as a barrier to release into the supernatant, we do not believe this is the case for two reasons. First, multiple time points were used for the assay and included times before, during, and after the HE assembly was "complete." Second, the relative permeability of the assembled envelope allows molecules of at least 10,000 Da through, as demonstrated by the dextran experiments. It is very likely that even larger molecules would pass the envelope prior to the final "tanning" process. An alternative explanation might be that the enzyme is bound so tightly to the assembling envelope that none escapes. Although this is possible, it is inconsistent with the reports in other systems and contradictory to the results of the DAB assays, which failed to demonstrate localization in the HE.

Ovoperoxidase from sea urchins is reported to have oxidoreductase activity requiring Mn^{+2} ions and certain phenols related to the respiratory burst (Turner *et al.*, 1985; 1986; 1988; Heinecke and Shapiro, 1989). Phenol oxidase in crustaceans oxidizes diphenols to the corresponding quinones that react in the tanning of crustacean exoskeletons to cross-link proteins within the chitin matrix (Stevenson, 1985). Perhaps the oxidase of the shrimp embryo is a closer analog of phenol oxidase than of ovoperoxidase. Pillai and Clark (1990) report that carbohydrates similar to those found in chitin are abundant in the peneaoid hatching envelope. Lectin-binding assays demonstrate the presence of oligosaccharides, especially mannose and *N*-acetylglucosamine, in the HE (Pillai and Clark, 1990; Lin, 1992; Glas, 1994). These sugars are the major components of chitin. A phenol-oxidase-driven cross-linkage of carbohydrates within the HE would be consistent with the presence of an oxidase. The action of such an enzyme is also consistent with the thinner HE in the presence of the oxidase inhibitors ATA and sodium sulfite.

Thus, an oxidase is probably involved, as indicated by the morphology of the HE after exposure to the peroxidase inhibitors ATA and sodium sulfite and the staining with DHTMR. The active enzyme is not similar to the ovoperoxidase identified in the sea urchin, as shown by the lack of localization with DAB or quantitation by the guaiacol assay; neither does the enzyme appear to be analogous to the enzyme that stimulates the peroxidatic reaction in fish fertilization. Nevertheless, a peroxidase-inhibitor-sensitive enzyme is involved with the assembly of the penaeoid HE. Further identification of such an active enzyme is required for a full understanding of the mechanisms responsible for HE assembly, elevation, and function.

Acknowledgments

The authors would like to express their appreciation to Dr. Wallis Clark, Jr., and his students at University of California, Bodega Marine Laboratory, for procurement of the *Sicyonia ingentis* and use of laboratory space. We are also grateful to Jim Hanifen and his colleagues at Louisiana Department of Wildlife and Fisheries for providing the *Trachypenaeus similis*. We thank Becky Demler, Ron Bouchard, Cindy Henk, and Dr. Sharon Matthews of the Louisiana State University Life Sciences Microscopy Facility for their assistance. Research supported by Louisiana Sea Grant #NA89-AA-D-SG226 project #R/SA-1 to JWL and JDG and Sigma Xi Grant-in-Aid of Research to PSG.

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